Bioconjugate Chemistry

Production and Characterization of a Camelid Single Domain Antibody−Urease Enzyme Conjugate for the Treatment of Cancer

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S Supporting Information

ABSTRACT: A novel immunoconjugate (L-DOS47) was developed and characterized as a therapeutic agent for tumors expressing CEACAM6. The single domain antibody AFAIKL2, which targets CEACAM6, was expressed in the *Escherichia coli* BL21 (DE3) pT7-7 system. High purity urease (HPU) was extracted and purified from Jack bean meal. AFAIKL2 was activated using *N*-succinimidyl [4-iodoacetyl] aminobenzoate (SIAB) as the cross-linker and then conjugated to urease. The activation and conjugation reactions were controlled by altering pH. Under these conditions, the material ratio achieved conjugation ratios of 8−11 antibodies per urease molecule, the residual free urease content was practically negligible (<2%), and high purity (>95%) L-DOS47 conjugate was produced using only ultradiafiltration to remove unreacted antibody and hydrolyzed cross-linker. L-DOS47 was characterized by a panel of analytical techniques including SEC, IEC, Western blot, ELISA, and LC-MS[E] peptide mapping. As the antibody−urease conjugate ratio increased, a higher binding signal was observed. The specificity and cytotoxicity of L-DOS47 was confirmed by screening in four cell lines (BxPC-3, A549, MCF7, and CEACAM6-transfected H23). BxPC-3, a CEACAM6-expressing cell line was found to be most susceptible to L-DOS47. L-DOS47 is being investigated as a potential therapeutic agent in human phase I clinical studies for nonsmall cell lung cancer.
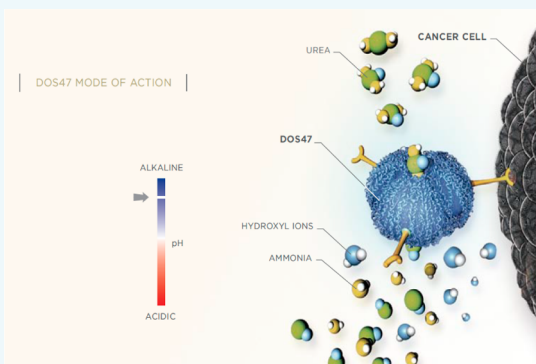
INTRODUCTION

The effectiveness of the most commonly used cancer chemotherapeutic drugs is limited by their narrow therapeutic indices and lack of selective effects on tumor cells. Antibody−drug conjugates (ADC) are being developed as target drug delivery systems for cancer therapy. ADCs have the advantage of reducing nonspecific side effects as drug is specifically delivered to the tumor site. ADCs are composed of an antibody and a cytotoxic drug conjugated via a linker. Antibody binds to the tumor cell surface antigen followed by its internalization into the cell where the linker is cleaved or degraded to release cytotoxic drug. To achieve a selective cytotoxic action, ADC must meet the following conditions: (1) a specific tumor antigen must be identified, (2) ADC must be able to internalize as most small molecule based cancer drugs act intracellularly, and (3) the chemistry of the linker must allow it to break down inside the cell to release the drug.[1] Several ADCs are under clinical assessment for the treatment of cancer. For instance, Brentuxiamab vedotin (Adcetris) has been approved by FDA for the treatment of Hodgkins lymphoma.[2] On the other hand, antibody directed enzyme prodrug therapy (ADEPT) employs a two-step approach. ADEPT improves selectivity by delivering antibody−enzyme conjugates to tumor sites where they bind to tumor associated antigens while the remaining unbound conjugates are eliminated from the bloodstream.[3] Once the antibody−enzyme conjugates accumulate, prodrug is adminis-

tered and is converted at the tumor site to its active cytotoxic form by the enzyme portion of the antibody−enzyme conjugate, thus achieving selective tumor cell death. Since the ADEPT concept was introduced by Bagshawe in 1987,[3−5] researchers have applied variations of this concept to develop more potent and specific anticancer therapeutics.[5−7]

We have developed a novel ADEPT approach to selectively killing tumor cells that does not require administration of a prodrug for cytotoxic activity. The antibody−enzyme conjugate contains an antibody that selectively binds to the cell surface CEACAM6 antigens and a urease enzyme moiety that converts endogenous urea into ammonia in situ to induce cytotoxicity. In this paper, we describe the manufacturing and character-ization of L-DOS47, a chemical conjugate of a recombinant single-domain antibody and Jack bean urease with a conjugation ratio of 10 antibodies per native urease molecule. Unlike conventional ADEPT, in which prodrugs are adminis-tered systemically, the L-DOS47 immunoconjugate uses urea, which is naturally abundant in tumor tissues, as the prodrug. However, given the large size of Jack bean urease (544 kDa), consideration was made to select a small single-domain but cancer-specific antibody. The original AFAI antibody was isolated by panning a naive phage display library of camelid

single-domain antibodies against the lung carcinoma A549[8,9] and showed immunoreactivity mainly to malignant tumors and particularly to lung adenocarcinomas.[9] The protein sequence of the AFAI antibody was later optimized to yield the proper pI for chemical conjugation purposes, and the optimized antibody was designated AFAIKL2. The small molecular size of the AFAIKL2 single-domain antibody (13 kDa) provides a size advantage over conventional immunoglobulins (~150 kDa), especially when multiple antibodies are needed to boost avidity of the antibody–urease conjugate. Besides, the camelid single-domain antibody has other favorable properties as compared to conventional immunoglobulins.[10−12] For instance, they are easy to clone and express recombinantly,[13,14] are generally more thermally and chemically stable,[15,16] and have improved epitope specificities.[17] Selective binding of the AFAIKL2 antibody to tumor associated antigen CEACAM6[8] on target tumor cells results in the accumulation of urease and subsequent hydrolysis of extracellular urea to produce ammonia, which is cytotoxic and creates an alkaline environment unfavorable to cancer cells.[18] Because of the complexity and the size of the conjugate, which can have a molecular weight up to 680 kDa, the conjugation chemistry, reaction, and separation procedures were developed to address the challenges in large-scale production. Because urease has multiple potential conjugation sites for the selected antibody, the conjugation ratio of L-DOS47, which is essential to drug potency, was characterized using an Experion SDS microchannel gel electrophoresis system.[19,20] L-DOS47 conjugate purity was determined by size exclusion chromatography. The chemical identity of L-DOS47 was characterized by mass spectrometric peptide mapping and Western blot. Because a primary amine (K32) is carried on the CDR3 region of the single-domain antibody, the distribution of the conjugation sites at the antibody side was determined by RP-HPLC and MALDI mass spectrometry. The conjugation sites for both the antibody and urease sides were also characterized by ESI mass spectrometry. The effect of conjugation ratio on the affinity of L-DOS47 binding to CEACAM6 was evaluated by ELISA. In vitro and in vivo studies were performed to confirm L-DOS47 binding and its ability to cause cytotoxicity in CEACAM6-expressing cancer cell lines.

RESULTS AND DISCUSSION

Jack bean urease is a hexameric enzyme consisting of six identical subunits of approximately 91 kDa each with 15 unbonded cysteine residues per subunit. The AFAIKL2 antibody contains seven primary amines and a disulfide bond. The primary amines on the antibody and the cysteine residues on the urease are the bases for chemical conjugation through a heterobifunctional cross-linker. However, the molecular size of the conjugate and the nature of the two proteins created challenges in scale-up production, purification, and characterization of the conjugate product. The immunoconjugate must be soluble and stable in aqueous media near physiological pH for use as parenteral drug; therefore, the isoelectric point (pI) of the recombinant antibody required careful sequence design because urease is extracted from a plant source and its pI (observed pI 4.8−5.1) could not be altered. Optimization to ensure reaction uniformity and removal of residual reactants and side products was critical to conjugation chemistry. Although several cross-linkers[7,22] are widely used for protein conjugations and were screened during development, N-succinimidyl [4-iodoacetyl]-aminobenzoate (SIAB) was chosen

for L-DOS47 conjugate production because of the differences in optimum pH for the two cross-linking reactions. During production, the antibody is activated with the cross-linker at pH 7.0 then buffer exchanged to pH 6.5 and mixed with urease. The antibody is then linked to urease by increasing the pH of the reaction media to 8.3. Because the reaction rate linking activated AFAIKL2 to urease is very low at pH 6.5, material uniformity in a large reaction vessel is ensured by premixing at pH 6.5. Therefore, the distribution of residual free urease and the subspecies of urease−(Ab)$_x$ are solely determined by probability and material molar ratios. At conjugation ratios of 8−11 antibodies/urease, the residual urease content is theoretically negligible and the L-DOS47 conjugate can be purified using ultradiafiltration to remove unreacted antibody and hydrolyzed cross-linker. Size exclusion chromatograms of L-DOS47 conjugate, free AFAIKL2, and free urease are shown in Figure 1.
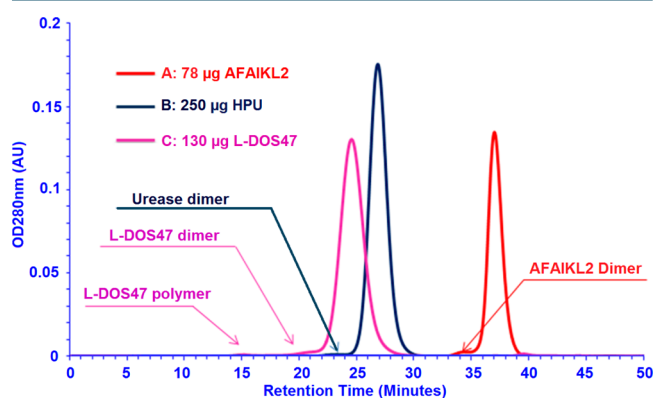

Figure 1. Size exclusion chromatograms of AFAIKL2 (A), high purity urease (B), and L-DOS47 conjugate (C). A very small dimer peak for each constituent appears in front of the respective monomer peaks. Formulation blank contains 10 mM histidine, 1% sucrose, 0.2 mM EDTA, pH 6.8.

The L-DOS47 conjugate elutes at ~24.6 min, the dimer (possibly linked by a disulfide bond or by an AFAIKL2 molecule activated with two SIAB) elutes as a small unresolved peak at 20.5 min, and the polymer elutes as a trace peak at the void time (~15 min). A trace peak at ~40 min represents the buffer components. The L-DOS47 conjugate peak width is slightly larger than but comparable to that of free urease, suggesting an evenly distributed conjugation reaction. Free antibody elutes at 37 min. A small unresolved peak at the front of the antibody peak represents noncovalent dimer. High purity of the antibody (>95%) is typically observed in the production lots, with its high molecular weight species including dimers not exceeding 5%. HPU typically elutes with a major peak at ~26.9 min, a small dimer peak at 24 min, and a trace polymer peak at 15 min. Purification of crude urease to HPU resulted in ~97% monomer, while the sum of dimer and polymer is not more than 3%. Greater than 95% L-DOS47 purity is typically achieved from simple purification using only ultradiafiltration. Because the SEC is run under native conditions, it can determine changes in effective molecular weights due to degradation and dissociation of protein quaternary structures; therefore, this method is also used as a stability indicating assay for monitoring L-DOS47 degradation during its shelf life. The presence of residual free urease in L-DOS47 was evaluated using ion exchange chromatography (Figure 2).
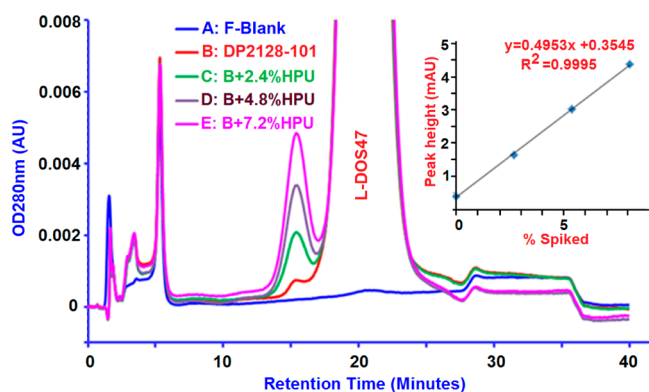
Figure 2. Ion exchange chromatograms of formulation blank (A), L-DOS47 (B), and L-DOS47 spiked with 2.4% (C), 4.8% (D), and 7.2% (E) HP urease (HPU). Formulation blank contains 10 mM histidine, 1% (w/v) sucrose, 0.2 mM EDTA, pH 6.8.

Figure 3. Snapshot of an Experion SDS window. (Panel 1) Overlay of electrophoregrams of lanes 1 and 4. (Panel 2) Lane L, molecular weight (MW) ladder; lanes 1, 2, 7, and 8, L-DOS47 produced with activated AFAIKL2 that had undergone additional IEC purification; lanes 3−6, L-DOS47 produced with AFAIKL2 that had not undergone additional IEC; lanes 9 and 10, HPU. In Panel 1, the numbers 2−14 on the x-axis are the peak numbers of the electrophoregram from lane 4; 3* represents the lowest MW marker peak, and 14* is the highest MW marker peak for the internal MW standard.

The residual free urease elutes at 15.33 min, which is very close to the spiked urease peak (15.42 min). The HPU peak is fairly well resolved from the large L-DOS47 peak (R_s of 1.48). As shown in the inset of Figure 2, the standard addition method (L-DOS47 spiked with 2.4%, 4.8% and 7.2% w/w HPU) to determine residual urease content exhibits good linearity (R^2 of 0.9995). The calculated residual urease content of this sample is 0.72%, and residual urease has not exceeded 2% in all production lots to date, demonstrating that the residual urease is practically negligible under these conditions and the manufacturing process does not require an additional step to separate the L-DOS47 conjugate from unconjugated urease.

During L-DOS47 production, each of the six monomeric urease subunits can be conjugated with zero, one, two, three, or four AFAIKL2 molecules; therefore, under denaturing conditions, SDS-Experion of L-DOS47 produces a pattern of multiple discrete peaks/bands from ∼90 to 155 kDa. Additionally, during the antibody activation reaction, a small portion of the antibody can be randomly activated with two SIAB per antibody molecule (AFAIKL2-(SIAB)$_2$), which results in conjugation of each of those antibody molecules to two subunits of urease. The one-antibody-two-subunits consequently produces a smaller second set of poorly resolved peaks/bands ranging from 200 to 260 kDa.

In Figure 3, Panel 2 depicts a virtual gel image and Panel 1 contains an overlay of the electrophoregrams from lanes 1 and 4. The L-DOS47 bench scale sample in lanes 1−2 and 7−8 was produced with activated AFAIKL2 that had been purified by ion exchange chromatography before the second reaction of the conjugation. Because the AFAIKL2-(SIAB)$_2$ species was removed, the resulting conjugate lacked the intercross-linked subunits and only a single set of peaks/bands was observed. In the L-DOS47 sample in lanes 3−6, the AFAIKL2 had been used directly in conjugation after the activation step without additional purification and consequently the small second set of peaks/bands is present. Lanes 9 and 10 were overloaded with an HPU sample.

The peak areas (Figure 3, Panel 1) and band intensities (Figure 3, Panel 2) depend on the relative abundances of urease subunits linked with the corresponding number of antibody molecules. The peak areas are integrated by the software after baseline correction, and the L-DOS47 conjugation ratio (CR) is calculated as follows (see Figure 4, representing lane 1 of the gel from Figure 3):
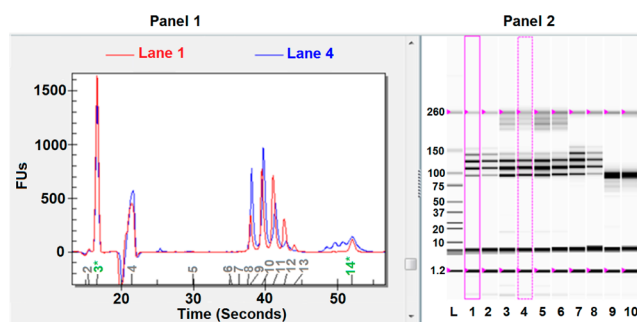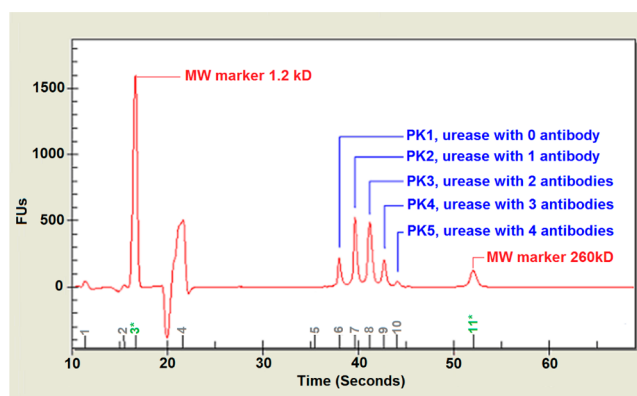

Figure 4. Electropherogram of L-DOS47 (lane 1 from Figure 3) showing the discrete peaks for urease subunits linked with 0−4 antibody molecules. The numbers 1−11 on the x-axis are the peak numbers; 3* represents the lowest MW marker peak, and 11* is the highest MW marker peak for the internal MW standard.

$$CR = 6*((PK_1*0 + PK_2*1 + PK_3*2 + PK_4*3 + PK_5*4)$$
$$/(PK_1 + PK_2 + PK_3 + PK_4 + PK_5))$$

Where PK_i (i =1−5) is the peak area of the urease subunit linked with i-1 antibody molecules.

While the AFAIKL2-(SIAB)$_2$ species could theoretically generate dimer or polymer conjugates by linking to two subunits from different native urease molecules, the minimal levels (less than 3%) of combined dimer and polymer peak areas observed in the size exclusion chromatogram of L-DOS47 sample (Figure 1) suggest that most of the AFAIKL2-(SIAB)$_2$ species contribute to intersubunit linkage of a single native urease molecule to produce monomer L-DOS47 and not to intermolecular linkages to produce dimer and polymer conjugates.

Analysis of L-DOS47 by dual Western blotting (Figure 5) confirms the banding pattern seen via SDS-Experion. The inset box shows an enlargement of the boxed region of the main blot in which the urease and conjugated species (N1−N4 corresponding to urease with 1−4 conjugated AFAIKL2, respectively) are labeled. When probed with an antiurease antibody, the 91 kDa urease band is the most strongly
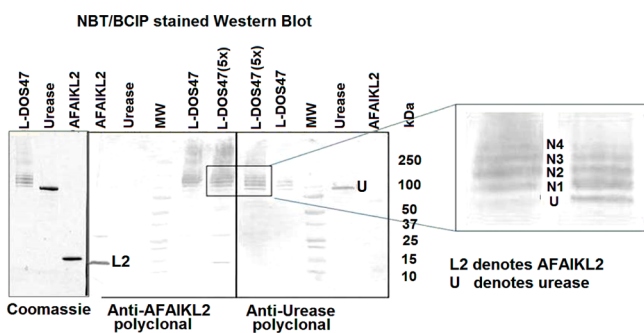
Figure 5. Western blot of AFAIKL2, urease, and L-DOS47. Left panel: Gel electrophoresis of L-DOS47, urease, and AFAIKL2 (Coomassie blue stained). Middle panel: Western blot of AFAIKL2, urease, and L-DOS47 (standard load and 5× overload) probed with anti-AFAIKL2 antibody. Right panel: Western blot of AFAIKL2, urease, and L-DOS47 (standard load and 5× overload) probed with antiurease antibody. Inset: Magnification of L-DOS47 bands.

visualized and the intensity of the higher molecular weight bands (corresponding to more highly conjugated species) decreases. In contrast, when probed with an anti-AFAIKL2 antibody, a 91 kDa band which is due to nonspecific adsorption is barely visible and the next two higher molecular weight bands (N1 and N2) are the most intense and dominant bands in the conjugate. The ability of L-DOS47 to be visualized by both the anti-AFAIKL2 and antiurease antibodies demonstrates the presence of both species in the conjugate.

The conjugation ratio of L-DOS47 is critical to its binding affinity for CEACAM6, the tumor antigen targeted by the antibody. L-DOS47 with different conjugation ratios (1.8–12 AFAIKL2 per urease) were produced by adjusting the AFAIKL2/HP urease molar ratios to evaluate the effect of conjugation ratio on binding affinity. The binding affinity of L-DOS47 to immobilized CEACAM6-A was found to be directly proportional to the number of antibodies conjugated to urease (Figure 6). With more antibodies conjugated, higher binding signals were observed. However, the effect was less pronounced at conjugation ratios of 6 or more.

Different L-DOS47 binding profiles were observed among BxPC-3 and A549 cell lines, which express varying levels of endogenous CEACAM6[23−25] (Figure 7). The results showed that L-DOS47 bound well to the pancreatic cell line BxPC-3,
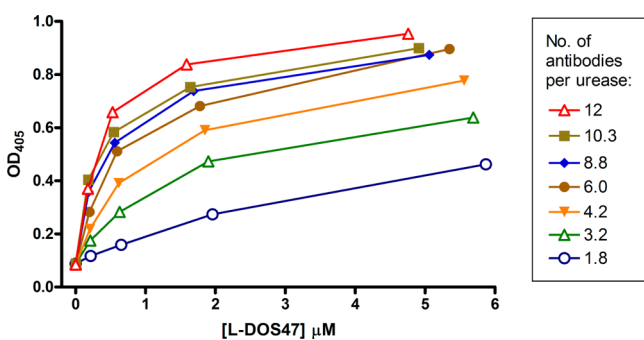

Figure 6. Effect of conjugation ratio on the binding activity of L-DOS47. L-DOS47 was prepared with different antibody conjugation ratios (from 1.8 to 12 antibodies per urease). Direct binding of the L-DOS47 samples to immobilized CEACAM6-A molecules was determined. The results represent the mean (n = 3) of representative experiments. The standard deviation (SD) was less than 10% for all values.
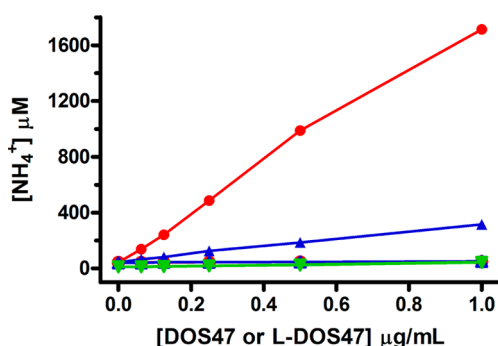

Figure 7. Direct binding of L-DOS47 to cancer cell lines BxPC-3, A549, and MCF7. The binding signal was represented by the amount of ammonia generated upon incubation with 20 mM urea. Elevated L-DOS47 binding was observed in BxPC-3 (solid red circles), while moderate binding was observed in A549 cells (solid blue triangles) and no binding was found in MCF7 cells (solid green inverted triangles). In addition, no binding was found with the unconjugated urease control in the corresponding cell lines (open red circles, open blue triangles, open green inverted triangles), suggesting that L-DOS47 binding was specific to BxPC-3 and A549 cells. The results represent the mean (n = 3) of representative experiments. The standard deviation (SD) was less than 10% for all values.

indicating that CEACAM6 antigen was better expressed on the cell surface. Moderate binding was observed in the lung cell line A549, but no binding was found in the breast cell line MCF7. The AFAIKL2 antibody, when conjugated to the urease enzyme, provided specific targeting toward CEACAM6-expressing cells. This was confirmed by the absence of binding signal in BxPC-3 and A549 cells treated with the unconjugated urease control.

BxPC-3 cells were very susceptible to L-DOS47 cytotoxicity (Figure 8), as shown by the rapid drop in cell survival when the cells were treated with less than 1 μg/mL of L-DOS47 and 20 mM urea. Moderate cytotoxicity was observed in A549 cells, and no effects were found in MCF7, which were consistent
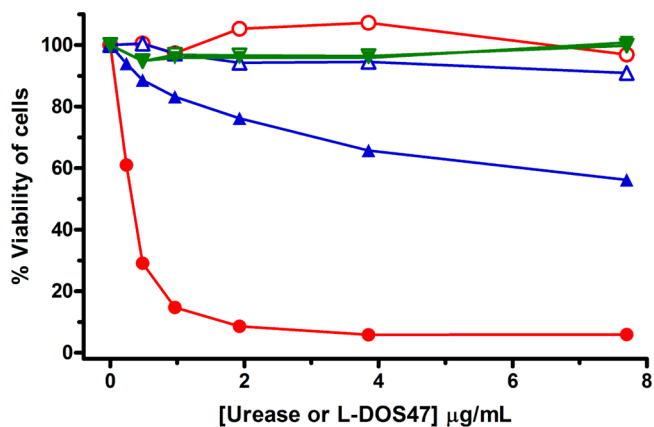

Figure 8. L-DOS47 induced cytotoxicity on BxPC-3 and A549 cells upon addition of 20 mM urea. No effects were observed in MCF7 cells (solid green inverted triangles). BxPC-3 (solid red circles) was highly susceptible to L-DOS47, whereas only moderate effects were observed in A549 cells (solid blue triangles). In addition, no binding was observed with the unconjugated urease control to the corresponding cell lines (open red circles, open blue triangles, open green inverted triangles). The results represent the mean (n = 3) of representative experiments. The standard deviation (SD) was less than 10% for all values.

with the results of the binding study. In addition, the urease negative control had no cytotoxic effect on any of the cell lines.

The functional role of CEACAM6 was confirmed by transfection of the CEACAM6 gene into H23 cells, which do not express CEACAM6 on the cell surface (Figure 9A). After
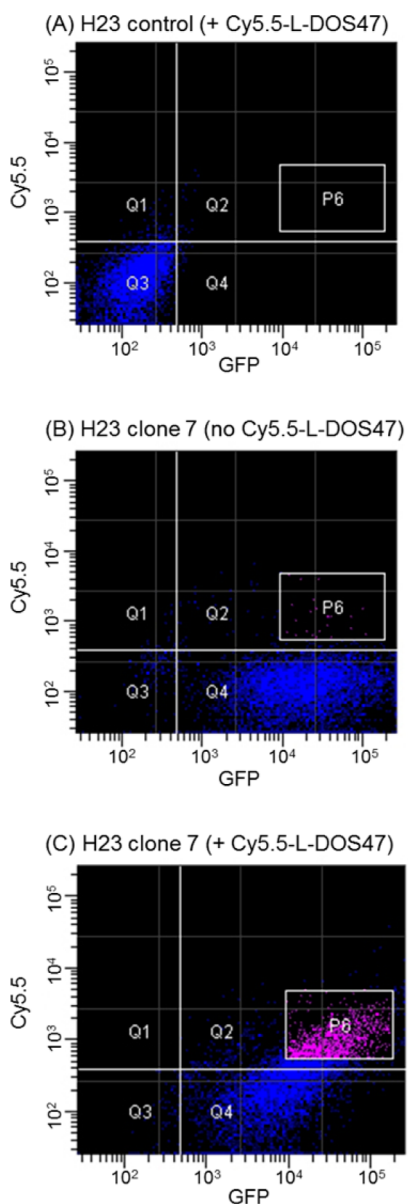

Figure 9. Flow cytometry analysis of CEACAM6 expression in CEACAM6-transfected H23 cell line. The transfected cells (clone 7) coexpress GFP from the same mRNA as CEACAM6. Cells were sorted directly after incubation with Cy5.5 labeled L-DOS47. (A) Untransfected H23 control cells incubated with Cy5.5-L-DOS47. (B) CEACAM6-transfected cells. (C) CEACAM6-transfected cells after incubation with Cy5.5-L-DOS47. P6 represents transfected cells with Cy5.5 fluorescence, corresponding to CEACAM6 expression on the cell surface.

transfection with the CEACAM6 gene, clones with CEACAM6 expressed on the cell surface (clone 7) were sorted with incubation of Cy5.5-L-DOS47 (Figures 9B, C). The H23 control cells showed no GFP or Cy5.5 fluorescence. The majority of the cells fell within Q3 with a mean intensity of 163, whereas the CEACAM6-transfected cells showed a shift in

average GFP intensity to 35000. After incubation with Cy5.5-L-DOS47, 35% of the transfected cells exhibited Cy5.5 fluorescence (P6), indicating that CEACAM6 was expressed on the cell surface. A direct binding study of the CEACAM6-transfected H23 cells showed that while H23 clone 7 expressed less CEACAM6 on the cell surface than A549 and BxPC-3 cells (Figure 10A), this CEACAM6-transfected clone was even more
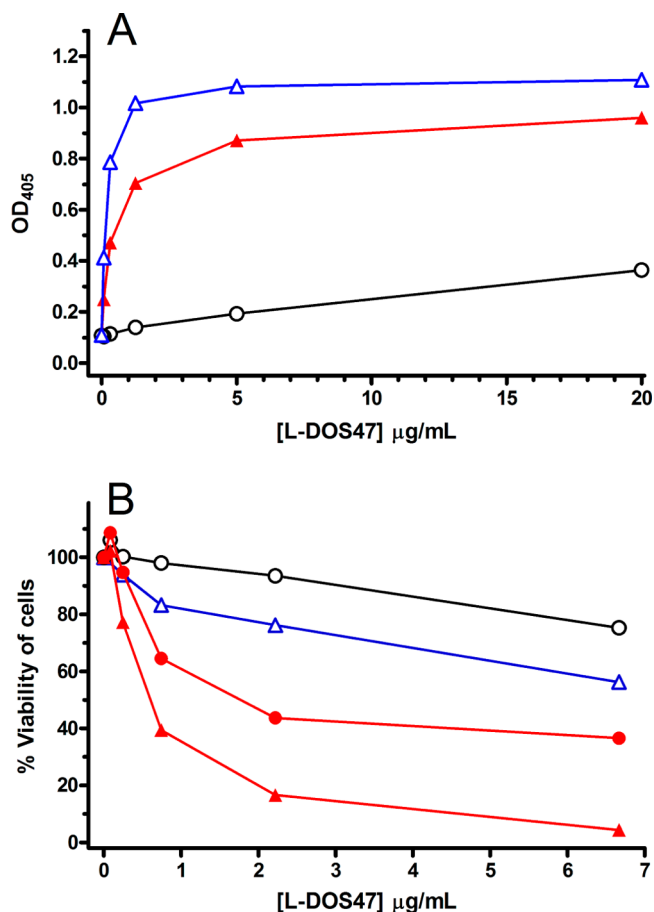

Figure 10. (A) Binding of L-DOS47 to CEACAM6-transfected H23 cells. The population of the transfected cell line (solid red triangles) was enriched by FACS cell sorting. The binding profile as compared to that of the native H23 cells (open black circles) and A549 cells (open blue triangles) showed that CEACAM6 was expressed in the transfected cells at a level lower than that of A549 cells. (B) Cytotoxicity assay of L-DOS47 on CEACAM6-transfected H23 cells. CEACAM6 overexpression in H23 cells (solid red triangles) greatly enhanced their susceptibility to L-DOS47 cytotoxicity as compared to BxPC-3 (solid red circles), A549 (open blue triangles), and native H23 (open black circles) cells after incubation with 8 mM urea. The results represent the mean ($n = 3$) of representative experiments. The standard deviation (SD) was less than 10% for all values.

susceptible to L-DOS47 cytotoxicity than BxPC-3 cells (Figure 10B) due to its high susceptibility to ammonia toxicity (Supporting Information Figure S4).

In addition, the in vivo activity of L-DOS47 on tumor cells was examined using the BxPC-3 xenograft model in nude mice. The results showed that L-DOS47 was effective in inhibiting tumor growth at doses as low as 7 μg/kg, and the effect was similar to treatment with 12.5 mg/kg of paclitaxel (Figure 11).

Because L-DOS47 is a chemical conjugate of AFAIKL2 and urease, tryptic peptides from both the antibody and the urease
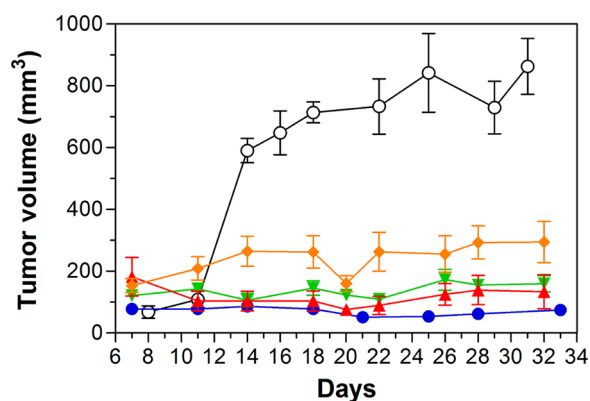
Figure 11. L-DOS47 inhibits BxPC-3 xenograft tumor growth in nude mice. Nude mice were subcutaneously injected with 5×10^6 cells suspended in 100 μL of Matrigel on the left shoulder. Vehicle and L-DOS47 were administered on days 14, 18, and 22 post tumor implantation, while the positive control, paclitaxel, was injected on days 14, 18, 21, 25, and 28. Significant inhibition of tumor growth was observed in all three L-DOS47 treatment groups [7 (inverted solid green triangles), 35 (solid orange diamonds), and 175 (solid red triangles) μg/kg] and paclitaxel control (solid blue circles) as compared to the vehicle-treated group (open black circles). Values are means ($n = 5$) ± SEM.

Figure 12. RP-HPLC chromatogram at 420 nm of a tryptic digest of AFAIKL2−Cys-FL. The identified conjugated peptides and their peptide masses are denoted at the corresponding HPLC peaks.

distribution of each activation site (Table 1) was calculated according to its peak area and the sum of all the identified HPLC peak areas in Figure 12.

Table 1. HPLC Peak Area and Distribution Percentage of Each Activation Site on AFAIKL2

Lys$_{76}$(L2K$_{76}$)		Lys$_{44}$ (L2K$_{44}$)		Met$_1$(L2M$_1$)		Lys$_{32}$ (L2K$_{32}$)	
area	%	area	%	area	%	area	%
185	35	107	20	140	26	97	18

enzyme as well as covalently cross-linked peptides of both proteins should be detected from tryptic digests of the conjugate. ESI LC-MS^E peptide mapping was performed to characterize the conjugate. As shown in the associated content (Figures S1−S3 and Tables S1−S3), peptides from AFAIKL2 appeared in the L-DOS47 spectrum but not in the HP urease spectrum. From the tryptic digests of L-DOS47, the peptide coverages for both the AFAIKL2 and urease amino acid sequences were 100% with a typical mass error of less than 6 ppm, and each of the peptides was confirmed by its MS^E MS/MS b/y fragment ions with mass errors of less than ±15 ppm.

To identify the activation sites of AFAIKL2 and to determine the distribution of each conjugation site, AFAIKL2 was activated using SIAB then conjugated to fluorescein labeled cysteine (Cys-FL). After trypsin digestion of the resulting AFAIKL2−Cys-FL and separation by RP-HPLC chromatography, the peak fractions were collected for MALDI-MS to identify the Cys-FL linked antibody tryptic peptides. Because only the SIAB activated sites can be linked to Cys-fluorescein, for which the maximum absorption wavelength in 0.025% TFA is 420 nm, only activated peptide peaks should be detected at 420 nm. For example, if lysine no. 32 of AFAIKL2 is activated by SIAB, it should be linked to Cys-FL and this tryptic digestion site will be missed during tryptic digestion; therefore, a peak with a molecular mass of 2768.113 Da should be observed which represents the Cys-FL linked lysine-in-middle peptide, (LSCAAHDPIFDK$_{32}$NLMGWG)−Cys-FL, denoted as L2K$_{32}$−Cys-FL. The RP-HPLC chromatogram of a tryptic digest of AFAIKL2−Cys-FL is shown in Figure 12.

The identified conjugated peptides with detected mass values are also labeled at the corresponding HPLC peaks in Figure 12. According to the amino acid sequence of the antibody, the primary amines from the six lysine residues and the N-terminal amine are theoretically available for the activation reaction. However, in practice, only four of them were substantially activated. This is most likely due to the tertiary structure of the antibody exposing those four primary amines on the surface while burying the others inside the native structure. The
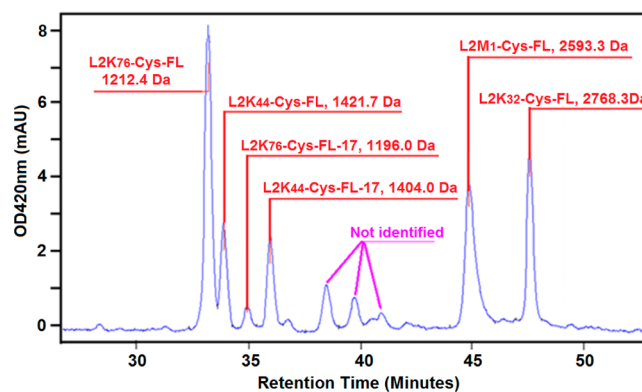
As shown in Table 1, the most active site of the antibody for the cross-linker is L2K$_{76}$, followed by L2M$_1$ and then L2K$_{44}$. L2K$_{32}$ is essential for antibody binding and is also the least active site but still contributed to ∼18% of total reactivity. For L-DOS47, the cross-linker activated AFAIKL2 was covalently linked to the cysteine residues exposed on the surface of the urease quaternary structure. Therefore, the covalently cross-linked peptides should be detected from the peptide spectrum of a tryptic digest of L-DOS47.

To identify those covalently cross-linked peptides, ESI LC-MS^E raw data of the tryptic digests from L-DOS47 samples were processed by BiopharmaLynx and searched against a variable-modifier library containing a set of user-created modifiers for all 15 cysteine residues on the urease side. According to the activation distribution in Table 1, those user created modifiers were the three lysine-in-middle peptides plus the linkage portion of SIAB $(C_9H_5O_2N, 159.0320 \text{ Da})$ (denoted as L2K$_{76}$, L2K$_{44}$, and L2K$_{32}$) and the N-terminal methionine plus the linkage (denoted as L2M$_1$). The results (detail in associated content) demonstrated that among the 15 cysteine residues of each urease subunit, only six were substantially conjugated. The most accessible cysteine is UC$_{824}$, followed in order by UC$_{663}$, UC$_{59}$, UC$_{207}$, UC$_{329}$, and UC$_{268}$. Cysteine residue Cys$_{592}$, which is essential to urease enzyme activity, was not substantially conjugated. The relative accessibilities of the four cross-linker activated AFAIKL2 sites to each of the six cysteine residues on the urease side were also different. For example, UC$_{329}$ was only accessible to L2M$_1$. Those substantial conjugation sites were also confirmed by their MS/MS fragment profiles. As an example, the conjugated peptide, L2K$_{32}$UC$_{663}$, whose sequence is (LSCAAHDPIFDKNLMGWGR)-linkage-(CDSSDNDNFR) and which has a peptide mass of 3517.4873, was identified with a mass match error of 2.1 ppm by searching it as CDSSDNDNFR a

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1    MDVQL QASGG GVVQP GGSLR LSCAA HDPIF DKNLM GWGRQ APGKQ REYVA   50
51   TISGS GGTNY ASSVE GRFTI SRDNA KKTVY LQMND LKPED TAVYY CNSAF   100
101  AIWGQ GTQVT VSSGG GEEDD GK                                    122
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Residues indicated in red are putative locations for attachment to urease

Figure 13. Amino acid sequence of AFAIKL2 antibody.

urease peptide modified with (LSCAAHD-PIFDKNLMGWGR)-linkage (2346.0674 Da) from the AFAIKL2 side as the modifier. The same peptide was also identified with a mass match error of 2.1 ppm by searching it as LSCAAHDPIFDKNLMGWGR, a AFAIKL2 peptide modified with the linkage-(CDSSDNDNFR) (1330.4520 Da) from the urease side as the modifier. The MS^E collision induced MS/MS spectrum of this conjugated peptide was mapped with 9 b/y fragment ions from the urease side by searching it as a urease peptide modified with the modifier from the AFAIKL2 side. The same spectrum was also mapped with 14 b/y ions from the AFAIKL2 side by searching it as an AFAIKL2 peptide with the modifier from the urease side.

CONCLUSION

We have successfully developed procedures for the conjugation and purification of the L-DOS47 immunoconjugate to be employed in its large-scale production. Using the established conjugation chemistry and reaction conditions, good uniformity was achieved by premixing the cross-linker activated antibody with the HP urease intermediate then adjusting the pH to activate the conjugation reaction. At conjugation ratios of 8−11 antibodies per urease molecule, the residual urease content was practically negligible and the L-DOS47 conjugate could be purified using ultradiafiltration to remove unreacted antibody and hydrolyzed cross-linker and avoiding a challenging step to separate residual urease from the final immunoconjugate. This procedure yielded a high purity L-DOS47 product (>95%) with free urease at less than 2%. The binding affinity of L-DOS47 to immobilized CEACAM6-A as evaluated by ELISA was directly proportional to the number of antibodies conjugated to urease and leveled off when the conjugation ratio was greater than 6. The apparent binding affinity of L-DOS47 as compared to the single domain antibody has been estimated by competitive ELISA to be 3.22 nM for L-DOS47 (MW = 622 kDa for a conjugation ratio of 6 antibodies to 1 urease) and 1.55 μM for AFAIKL2 (MW = 12.9 kDa) (Supporting Information Figure S5). The results suggested that the binding affinity of L-DOS47 is about 500 times that of the AFAIKL2 antibody. Conjugation ratios have ranged from 9 to 11 antibodies per urease molecule for all large-scale batches, demonstrating that optimum conjugation had been achieved under these conditions. The dual antibody Western Blot assay confirmed the chemical identity of the conjugate. ESI LC-MS^E peptide mapping analysis achieved 100% sequence recoveries for both the antibody and urease from the L-DOS47 immunoconjugate. Peptide sequences with more than three amino acid residues including the C-terminal and N-terminal sequences of both AFAIKL2 and urease were confirmed by MS/MS b/y fragment maps of the corresponding peptides. Effective conjugation sites (4 at the AFAIKL2 side and 6 at the urease side) were identified by ESI LC-MS^E peptide mapping analysis of L-DOS47 samples. Those cross-linked peptides were confirmed by MS/MS b/y fragment maps of the related peptides from both the antibody and the urease sides.

The specificity of L-DOS47 toward the two CEACAM6-expressing cell lines BxPC-3 and A549 was illustrated by the absence of binding and cytotoxic activities of unconjugated urease versus the antibody-conjugated L-DOS47 counterpart. Among the cell lines tested, BxPC-3 showed the strongest binding signal whereas A549 demonstrated moderate binding. The binding signal of BxPC-3 was about five times of that of A549. The results were consistent with those observed in the cytotoxicity assays. L-DOS47 induced a much higher cytotoxic effect on BxPC-3 than A549. No cytotoxic response was observed in MCF-7 and H23 cells due to the lack of L-DOS47 binding. Furthermore, the binding specificity and biological activity of L-DOS47 to the CEACAM6 antigen were demonstrated in H23 cells transfected with the CEACAM6 gene. Transfected H23 cells showed a significant increase in L-DOS47 binding as well as elevated susceptibility to its cytotoxic effects. An in vivo xenograft study also supported the theory that L-DOS47 was effective against tumor growth in CEACAM6-expressing cell lines such as BxPC-3. L-DOS47 is being investigated as a potential therapeutic agent in human phase I clinical studies for nonsmall cell lung cancer and immunogenicity studies in human are underway.

MATERIALS AND METHODS

Production of High Purity Urease Intermediate. Urease (referred to as crude urease hereafter) was procured from BioVectra Inc. (Charlottetown, PE, Canada). Prior to use in conjugation, crude urease was purified to remove Jack bean matrix protein contaminants such as canavalin and concanavalin A. Crude urease was dissolved in high purity water, and the pH was brought to 5.15 with 10 mM acetic acid, 0.2 mM EDTA (acetate-EDTA buffer), and then filtered under vacuum using a slurry of Celite 503. The filter cake was washed with 10 mM sodium acetate, 1 mM EDTA, pH 5.15, and dried under vacuum. The urease-containing filtrate was cooled to 0−4 °C and fractionated by adding chilled ethanol to a final concentration of 25% (v/v). The mixture was stirred for 15 min then filtered under vacuum using washed Celite 503. The filter cake was washed with acetate-EDTA buffer containing 25% (v/v) ethanol then dried under vacuum.

Filter cakes were resuspended in acetate-EDTA buffer, and the slurry was filtered through Celite under vacuum to collect the filtrate. The resulting filter cake was washed with acetate-EDTA buffer and dried under vacuum. The wash and the initial filtrate were filtered through a 0.65 μm capsule. This ethanol fractionated urease filtrate was concentrated about 2 times using two Sartorius Sartocon 100 kDa MWCO poly(ether sulfone) membranes followed by buffer exchange into acetate-EDTA buffer.

Imidazole and TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) were added to this medium purity urease at final concentrations of 20 mM and 1 mM, respectively, and the pH
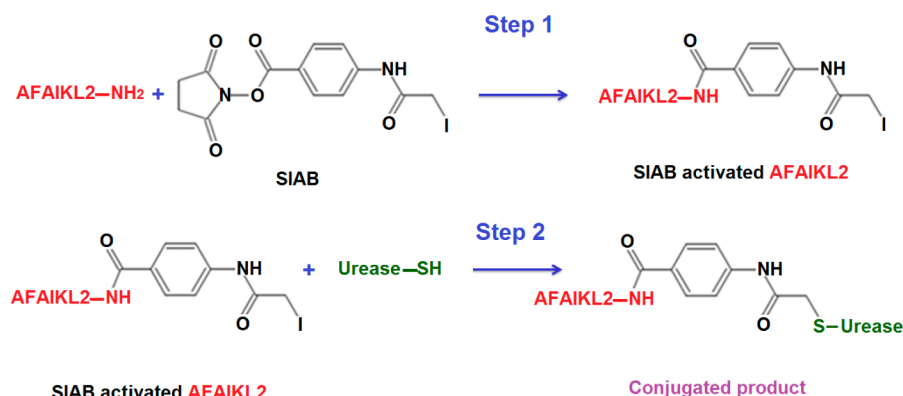
Figure 14. Synthesis of L-DOS47 conjugate product is a two-step reaction. Step 1 is an activation of antibody AFAIKL2 using SIAB, and step 2 involves conjugation of activated antibody with urease enzyme to form the antibody—urease conjugate L-DOS47.

was adjusted to 6.5. The protein solution was loaded onto a DEAE-Sepharose Fast Flow column pre-equilibrated with 20 mM imidazole, 1 mM TCEP, pH 6.5 (imidazole-TCEP buffer). All steps were performed at a flow rate of 500 mL/min. The column was washed with imidazole-TCEP buffer followed by imidazole-TCEP buffer containing 80 mM NaCl to remove unbound impurities. Urease was eluted with imidazole-TCEP buffer containing 180 mM NaCl. Fractions with A_{280} > 0.1 and purity by SEC of ≥90−97% were pooled.

The pooled fractions were concentrated to a target protein concentration of 6−8 mg/mL using two Sartorious Sartocon 100 kDa MWCO PESU membranes, then ultradiafiltered against acetate-EDTA buffer containing 10 mM sodium acetate, 1 mM EDTA, pH 6.5. The yield from this step is typically >55% of the starting activity.

Expression and Purification of AFAIKL2 Drug Intermediate. The amino acid sequence of the AFAIKL2 antibody is shown in Figure 13.

The antibody gene was expressed in the *E. coli* BL21 (DE3) pT7-7 system. One vial of the master cell bank was aseptically inoculated through three seeding steps into 350 L of Luria HiVeg (20 g/L) supplemented with 50 mg/L kanamycin, 1 g/L cerelose, 0.02 g/L MgSO$_4$, and 0.01% Biospumex antifoam reagent in a 500 L fermenter. The process was controlled to maintain the dissolved oxygen at >20%, the temperature at 37 °C ± 2 °C, the back pressure between 5 and 20 psi, the pH at 7.0 ± 0.2, the OD_{600} between 0.5 and 40, and the glucose concentration between 1 and 3 g/L. Once the culture reached an OD_{600} of 7−10, antibody expression was induced by the addition of IPTG to a final concentration of 1 mM and allowed to continue for 6−8 h. The cells were harvested by centrifugation, washed, and lysed to release the inclusion bodies, then resuspended in 10 volumes of 50 mM imidazole pH 6.8. The cell suspension was homogenized in batches, and the homogenate was passed first through a 75 μm stainless steel sanitary screen then through a microfluidizer with a minimum pressure of 10000 psi for a total of three passes while maintaining the temperature below 10 °C. The cell lysate was centrifuged, the insoluble material was pooled, and the pellet was resuspended with homogenization in 10 volumes of 1% Triton X-100 with 5 mM DTT. The washed pellet was collected by centrifugation. This step was repeated and followed by two washes with 25 mM sodium acetate, pH 4.0, containing 5 mM DTT to remove residual Triton X-100. The pellet was then adjusted to pH 4.

The pellet containing the washed inclusion bodies was resuspended in 8 M urea, 25 mM DTT, 125 mM sodium acetate, pH 4.0, and then solubilized alternately with a Ross homogenizer and an overhead mechanical mixer until there was no further change in visual appearance, then mixed for a total time of 3 h. The solubilized inclusion bodies were centrifuged, and the clarified supernatant was loaded at 550 mL/min onto a SP-Sepharose XL column that was pre-equilibrated with 8 M urea in 125 mM sodium acetate at pH 4.0 (SP equilibration buffer). After the clarified supernatant was loaded, the column was washed with equilibration buffer until the eluate A_{280} dropped below 0.05, followed by 8 M urea in 125 mM sodium acetate containing 50 mM NaCl, pH 4.0, until the eluate A_{280} dropped below 0.05. The pump speed was reduced to 275 mL/min, and 3 cv of 8 M urea containing 25 mM sodium acetate, 180 mM NaCl, at pH 4.0 were applied to the column to elute the AFAIKL2. Fractions with an A_{280} > 0.4 and an A_{280} /A_{260} ratio >1.5 were pooled and analyzed for purity and protein content. The percent yield from this step was typically 35−45%. The pooled material was diluted with SP equilibration buffer to ≤2.5 g/L, the pH was adjusted to 8.0 with 2 M Tris-Cl pH 8.0, DTT was added to 2.5 mM, and the conditioned pool was mixed for 60 min to fully reduce the denatured protein. The denatured protein solution was then diluted to a final protein concentration of less than 0.1 mg/mL in the refolding buffer containing 25 mM Tris-Cl, pH 8.5. The refolding was carried out at 2−8 °C and tracked by Ellman's assay and C18 reverse phase HPLC until the level of free sulfhydryl was <0.75 μM and only fully oxidized protein could be detected.

The refolded protein solution was loaded onto a Q-Sepharose XL column pre-equilibrated with 25 mM imidazole at pH 6.8, and the column was washed with equilibration buffer until the A_{280} was <0.05, followed with 25 mM imidazole at pH 6.8 containing 50 mM NaCl until the A_{280} was <0.01. The protein was then eluted with 25 mM imidazole, pH 6.8, containing 150 mM NaCl, and fractions were collected until the A_{280} was ≤0.3. Fractions were combined to create a target pool with not less than 97% purity and a yield of not less than 45%. The pool was then concentrated to 3−5 g/L using a UF/DF system with Hydrosart regenerated cellulose 5 kDa MWCO cartridges, followed by buffer exchange against 10 mM phosphate buffer pH 7.0 and lyophilization.

Conjugation Chemistry. The conjugation was carried out in two steps (Figure 14). In the first step, primary amine groups on AFAIKL2 were activated by reaction with the NHS ester portion of SIAB. In the second step, the activated AFAIK2 was

coupled to thiol groups on urease via the iodoacetamide end of SIAB.

Activation of AFAIKL2 Antibody. Lyophilized AFAIKL2 (25g) was dissolved in water for injection (WFI). SIAB (2.00 g) was dissolved in anhydrous DMF and added to the AFAIKL2 solution in three equal aliquots with 60 min of stirring after each addition. One hour after the final addition of SIAB, any remaining unreacted NHS groups were quenched by addition of a 10 times molar excess of glycine over the original amount of SIAB added. To remove the hydrolyzed and glycine quenched SIAB, the reaction solution was concentrated to 10 mg/mL AFAIKL2 using a Sartorious Sartocon 5 kDa MWCO followed by buffer exchange into 10 mM sodium acetate and 1 mM EDTA (pH 6.5).

Conjugation of Activated Antibody to Urease. High purity urease (50 g) was mixed with the activated AFAIKL2 in 10 mM sodium acetate and 1 mM EDTA (pH 6.5). The pH was then brought to 8.3 by the addition of 1 M sodium borate at pH 8.5, which allows the iodoacetyl group on the activated antibody to react with available cysteine residues on the urease. The reaction was allowed to proceed for 90 min with stirring. The unreacted iodoacetyl groups were then quenched by addition of 10 times molar excess of cysteine over the original amount of SIAB added, and the solution was mixed for 60 min. To remove unconjugated AFAIKL2, the L-DOS47 was concentrated to a target of 6 mg/mL using a 100 kDa MWCO Sartorious Sartocon followed by buffer exchange into 10 mM L-histidine, at pH 6.8, 0.2 mM EDTA. Sucrose was added to a final concentration of 1% w/v, and the L-DOS47 was diluted to a target concentration of 1.8 g/L with 10 mM L-histidine, pH 6.8, 0.2 mM EDTA.

Size Exclusion Chromatography (SEC) for Purity Evaluation. A Waters 2695 HPLC system with a 996 PAD was employed with Empower 2 software for data acquisition and processing. Chromatograms were recorded over 210−400 ± 4 nm, with the signal at 280 nm extracted for processing. Separation was performed on a Superose 6 100/300 GL column (GE). Proteins were eluted in 10 mM phosphate, 50 mM NaCl, 0.2 mM EDTA, at pH 7.2. Separation was carried out with an isocratic flow at 0.5 mL/min after injection of 100 μL of neat samples. The column was run at room temperature, while the sample temperature was controlled at 5 ± 2 °C.

Ion Exchange Chromatography (IEC) for Residual Urease. A Waters 2695 HPLC system with a 996 PAD and Empower software was employed. Chromatograms were acquired over 210−400 nm ± 4 nm, with the signal at 280 nm extracted for processing. The column (Mono-Q 5/50 GL, GE) was run at room temperature, while the sample temperature was controlled at 5 ± 2 °C. The elution buffers contained 50 mM acetate, 0.025% polysorbate 80 (super pure, HX2, NOF Corporation, Tokyo) with (buffer B) or without (buffer A) 0.70 M NaCl, pH 5.50. The column was equilibrated with 15% buffer B and 85% buffer A for 6 min at 1 mL/min flow rate (6 CV) before samples were injected. After a wash cycle (15% buffer B at 1.0 mL/min for 6 min), proteins were eluted by a gradient of 15−60% buffer B in 20 min with a 0.5 mL/min flow rate. After cleaning with 100% buffer B for 6 min at 1.0 mL/min, the column was re-equilibrated with 15% buffer B before the next sample injection. Then 800 μL of neat L-DOS47 samples were spiked with HP urease reference standard to final percentages of 0−8% w/w, and 50 μL/sample were injected. Peak height was used to calculate residual urease content using standard addition as the calibration method.

Experion SDS Microchannel Gel Electrophoresis for Determination of Conjugation Ratio. A Bio-Rad Experion automated electrophoresis system and a Bio-Rad SDS gel electrophoresis kit (Pro260 Kit) were employed to analyze L-DOS47 conjugation ratios. Samples were diluted with Tris-HCl buffer (10 mM, 0.2 mM EDTA, at pH 7.0) to a target protein concentration of 0.5 mg/mL, then 4 μL of diluted sample or molecular weight ladder was mixed with 2 μL of sample buffer and briefly centrifuged. Samples were heated at 70 °C for 10 min and then loaded onto the microchannel chip after the system, and channels were primed with gel-stain solution. Electropherograms were recorded automatically by the Experion software.

Western Blot for Identity Characterization. L-DOS47 test samples and AFAIKL2/HPU controls were resolved by SDS-PAGE gel electrophoresis then transferred to a nitrocellulose membrane using the Invitrogen iBlot system. Duplicate blots were made from gels run in parallel. Confirmation of AFAIKL2 identity requires detection using a rabbit anti-AFAIKL2 IgG primary antibody (Rockland) with secondary detection using a goat antirabbit IgG conjugated to alkaline phosphatase (AP) (Sigma). Confirmation of urease identity requires detection using a rabbit antiurease IgG primary antibody (Rockland Immunochemicals) with secondary detection using a goat antirabbit IgG conjugated to AP. For detection using anti-AFAIKL2 IgG, L-DOS47 samples were diluted to 2 μg/mL in TBS containing 0.1 mg/mL BSA and then mixed 1:1 with protein gel loading buffer, heated to 70 °C for 10 min, and 0.01 μg of L-DOS47 was loaded per lane. For detection using antiurease IgG, L-DOS47 samples were diluted to 20 μg/mL in TBS containing 0.1 mg/mL BSA then mixed 1:1 with protein gel loading buffer, heated to 70 °C for 10 min, and 0.1 μg of L-DOS47 was loaded per lane. Final development of the Western blots was performed with AP buffer containing NBT/BCIP.

ELISA of L-DOS47 with Different Conjugation Ratios. To study the effect of conjugation ratio on the affinity of L-DOS47 binding to its targeting antigen CEACAM6, L-DOS47 conjugates with different conjugation ratios were produced at bench scale by adjusting the AFAIKL2/HPU molar ratios during conjugation. The conjugation ratios of the resulting conjugates were determined by SDS-Experion, and the protein concentrations were determined by micro Lowry using a Total Protein Kit (Sigma). Microtiter plates were coated with 100 μL/well of the A domain peptide of the CEACAM6 antigen (CEACAM6-A, 2.5 μg/mL in PBS) and incubated at room temperature for 6 h. The plates were washed twice with buffer A (0.05% BSA in PBS), blocked with 150 μL/well 3% BSA/PBS at 4 °C overnight and then washed twice with buffer A. All subsequent steps were performed at room temperature with gentle shaking. L-DOS47 (100 μL/well) was added and incubated for 2 h, the plates were washed 3 times with buffer A and then 100 μL/well of antiurease IgG (1:12,000, Rockland) was added and incubated for 1 h. After three washes with buffer A, 100 μL/well goat antirabbit IgG-AP (1:6,000, Sigma) was added and incubated for 1 h. Then, after three washes with buffer A, 100 μL/well AP substrate solution was added and incubated for 25 min. Absorbance was determined at 405 nm.

Determination of Activation Sites on AFAIKL2 Antibody. To prepare fluorescein-labeled cysteine (Cys-FL), cysteine was reacted in excess with NHS-ester fluorescein (Pierce) in 1 M borate at pH 8.0 for 60 min at room

temperature. The reaction solution was separated by RP-HPLC with a C8 column. The Cys-FL peak fraction was identified by MALDI mass spectrometry, and its concentration was determined by spectrometry according to its extinction coefficient at 493 nm and pH 7. Peak fraction aliquots were lyophilized and stored in the dark at −20 °C. The AFAIKL2 antibody was first activated with the SIAB cross-linker at bench scale, and hydrolyzed SIAB was removed by a G25 desalting column prepared in-house. The activated antibody was allowed to react with the fluorescein-labeled cysteine (Cys-FL) in 100 mM borate buffer pH 8.3 for 90 min at room temperature. The reaction solution was buffer exchanged with 30 mM ammonium hydrogen carbonate by a G25 desalting column, and the resulting AFAIKL2−Cys-FL was diluted to 0.5−0.8 mg/mL in 30 mM ammonium hydrogen carbonate containing 20% acetonitrile. Trypsin (Promega) was added to a final protein to trypsin ratio of 20:1, and the digestion was carried out at 37 °C for 36 h. The resulting tryptic digest was reduced by adding 0.1 M TCEP to a final concentration of 2 mM and then separated by reverse phase HPLC (Agilent 1100 system with a Zobax 300SB-C18 column, 5 μm, 4.6 mm × 150 mm, gradient from 0 to 45% acetonitrile, 0.025% TFA in 55 min), and absorption was recorded at 420 nm. Peak fractions containing fluorescein modified peptides were collected and MALDI-mass spectrometry in Reflectron mode (Micromass Tof 2e) was applied. The HPLC peak areas of the corresponding peptides were used to calculate the distribution percentage of each activation site.

Peptide Mapping and Identification of Conjugated Peptides by ESI Mass Spectrometry. A Waters Xevo G2 QTOF mass spectrometer and an Acquity UPLC system H class with a BEH300 C18 column (1.7 μm, 2.1 mm × 150 mm) were employed. Each L-DOS47 sample (1.5−2.0 mg/mL, 50.0 μL) was mixed with 0.063 ± 0.003 g of guanidine-HCl. After the salt was fully dissolved, 1.50 μL of 0.7 M DTT was added and the solution was incubated at 60 °C for 30 min. Then 10.0 μL of 0.20 M iodoacetamide (IAA) was added, and the pH was adjusted to 8.0−8.5 with saturated Tris-Base solution. The sample was incubated at 37 °C for 60 min. Then 50.0 μL of each alkylated sample was mixed with 15.0 μL of 0.1 M CaCl$_2$ and 80.0 μL of Tris buffer (50 mM Tris-HCl, at pH 8.0), and then 3.00 μL of 0.5 mg/mL trypsin solution was added. The tryptic digestion was carried out at 37 °C for 20−24 h. After tryptic digestion, 50.0 μL of the digest was mixed with 0.50 μL of neat formic acid for LC-MS analysis.

The column temperature was set at 60 °C, and solvent A (0.075% v/v formic acid in water) and solvent B (0.075% formic acid in acetonitrile) were used for UPLC separation. The UPLC was performed with a flow rate of 0.15 mL/min with a gradient from 0 to 55% solvent B in 80 min.

LC-MS analysis was performed with Masslynx V4.1 software. LC-MS^E TIC (total ion counts) data acquisition was carried out in an m/z range of 50−2000 Da in resolution mode with a scan rate of 0.3/s, capillary voltage 3.0 kV, sample cone voltage 25 V, and extraction cone voltage 4.0 kV. The MS^E MS/MS fragmentation TIC data acquisition was performed with collision energy ramped from 20 to 40 V. Ion source temperature was set at 100 °C, and desolvation temperature was set at 300 °C. Desolvation gas flow was 600 L/h. A real time lock mass TIC raw data set (scan/20s) was acquired with 100 fmol/μL Glu-Fib B at a flow rate of 3.0 μL/min.

Mass spectrometric raw data were processed with Bio-Pharmalynx (v1.2) in peptide map mode with a resolution of

20000. A lock mass of 785.8426 Da was applied for real-time point-to-point mass calibration. The low energy MS ion intensity threshold was set at 3000 counts, and the MS^E ion intensity threshold was set at 300 counts. Mass match tolerances were set at 15 ppm for both MS and MS^E data sets. AFAIKL2 and urease amino acid sequences were input into the sequence library for peptide matching/identification. Variable modifiers including Deamidation N, Deamidation succinimide N, Oxidation M, Oxidation 2×M, +K, +Na, and a fixed modifier of Carbamidomethyl C (for alkylated cysteine) were applied for peptide map analysis. To identify the conjugated peptides on the AFAIKL2 side, the cysteine-containing peptides of urease plus the linkage section of cross-linker SIAB (C$_9$H$_5$O$_2$N, 159.0320 Da) were created as variable modifiers and included in the variable modifier library. In this case, Carbamidomethyl C was included as a variable modifier. To identify the conjugated peptides on the urease side, the lysine-in-middle peptides X$_n$KX$_m$ of AFAIKL2 plus the linkage section of SIAB were created as variable modifiers and included in the variable modifier library.

Cell Culture. Human breast (MCF-7), lung (A549 and H23), and pancreatic (BxPC-3) cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA). Cells were grown in advanced RPMI 1640 medium supplemented with 5% FBS, 2 mM Glutamax, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C in a humidified incubator gassed with 5% CO$_2$.

Transfection of CEACAM6 Gene to H23 Cells. A mammalian expression vector containing the G418 selectable marker was cloned with PMP-GFP (plasma membrane protein−green fluorescent protein) and CEACAM6 genes. Cellfectin reagent (Life Technologies) was used to transfect the expression vector into H23 cells. In brief, 2 × 10^6 cells/well of H23 cells in 2 mL of complete RPMI medium (containing 10% fetal bovine serum and 50 U/mL penicillin and 50 μg/mL streptomycin) were seeded in a 6-well culture plate and incubated at 37 °C overnight. Cellfectin reagent (10 μL) and DNA (1−2 μL) were diluted in 100 μL of serum-free RPMI medium, respectively. The two diluted solutions were then combined, mixed gently, and incubated at RT for 30 min. The plates were washed twice with 1.5 mL of serum-free RPMI medium. The combined solution was diluted in 0.8 mL of serum-free RPMI medium, mixed gently, and added to the cells. The cells were incubated at 37 °C and 5% CO$_2$ overnight. The next day, the medium was replaced with 2 mL of complete RPMI medium. The transfected cells coexpressed GFP from the same mRNA as CEACAM6. The transfected cells were selected by incubation in medium containing 400 μg/mL G418 antibiotic. Flow cytometry was performed in the University of Alberta Faculty of Medicine and Dentistry Flow Cytometry Facility. Cells were sorted after incubation with Cy5.5-L-DOS47.

Whole-Cell Binding Assay of L-DOS47. Cell monolayers were prepared by seeding 100 μL/well of tumor cells (4 × 10^4 cells/well) in 96-well culture plates and incubated overnight at 37 °C 5% and CO$_2$. The next morning, medium was removed from the plates and the cell monolayers were fixed with 100 μL/well of 0.05% glutaraldehyde (in phosphate buffered saline, PBS) for 10 min at room temperature (RT). The plates were then washed with PBS and 120 μL/well of glycine solution (50 mM) was added and incubated at 37 °C for 20 min. After incubation, the plates were blocked with 120 μL/well of 1% BSA/PBS at 37 °C for 30 min. Then the plates were washed

three times with buffer A (0.05% BSA in PBS), and 80 μL/well of diluted L-DOS47 or DOS47 solutions were added and incubated at 37 °C for 1.5 h. Binding signal was generated by either the urea or antibody method: (1) With the urea approach, the plates were washed four times with buffer A and 80 μL/well of 20 mM urea (prepared in 0.1 M phosphate buffer, at pH 7.6) was added and incubated at 37 °C for 30 min. After incubation, 40 μL/well of 1 N HCl was added to stop the reaction. The amount of ammonia produced in each well was determined using the indophenol assay.[21] In brief, solution A was freshly prepared by dissolving 165 mg of phenol and 132 mg of NaOH pellets in 10 mL of water, followed by adding 66 μL of sodium nitroprusside solution (10 mg/mL). Solution B was prepared by adding 40 μL of sodium hypochlorite to 5 mL of water. Sample solutions (30 μL each) from the whole-cell binding assay (see below) were transferred to a new 96-well plate containing 3.3 μL/well of 5N NaOH and 66.7 μL/well water. Solution A (50 μL/well) and solution B (50 μL/well) were added, and the plates were then transferred to a microplate reader for color development at 37 °C for 30 min. OD was measured at 630 nm. The amount of ammonia produced in each well was calculated from a calibration curve using ammonia chloride as standards (from 0 to 150 μM). (2) For the antibody method, endogenous peroxidase was first quenched with 100 μL/well of 0.3% hydrogen peroxide for 30 min before the blocking step. After incubation with the test articles, the plates were washed with PBS and diluted anti-AFAIKL2–peroxidase antibody conjugate (1:8000) were prepared in buffer A containing 0.05% Tween-20 and 0.1% skim milk powder. The plates were washed three times with buffer A and 100 μL/well of antibody–peroxidase conjugate was added to the plates. After incubating at 37 °C for 1 h, the plates were washed three times with buffer A. Peroxidase substrate was prepared at 1 mM in sodium citrate buffer containing 0.03% H_2O_2, and 100 μL/well of the substrate solution was added and incubated at RT for 30 min. The plates were transferred to a microplate reader for OD measurement at 405 nm.

Cytotoxicity Assay of L-DOS47. Cell monolayers were prepared by seeding 100 μL/well of tumor cells (4×10^4 cells/well) in 96-well culture plates and incubating overnight at 37 °C and 5% CO_2. Medium was then removed from the plates, and 80 μL/well of diluted L-DOS47 or HP urease were added and further incubated at 37 °C and 5% CO_2 for 2 h. After incubation, the plates were washed three times with KR-II buffer/0.05% BSA, and 100 μL/well of either 8 or 20 mM urea solution was added. The plates were incubated at 37 °C overnight. The next morning, medium was removed and replaced with 100 μL/well plain medium. Cell viability was determined using a MTS cell viability assay, in which a 20:1 v/v solution of MTS/PES (MTS, 2 mg/mL; PES, 1 mg/mL) was prepared and 20 μL/well of the mixture was added to the plates. The plates were then incubated at 37 °C and 5% CO_2 for 1 h, and OD was measured at 630 nm with reference at 490 nm.

In Vivo Animal Studies. Male nude mice (CD-1, Charles River Canada) were used as the animal model for xenograft development. The mice were injected subcutaneously in the left shoulder with a mix of 5×10^6 BxPC-3 human pancreatic cancer cells suspended in 100 μL of Matrigel. The tumor growth was steady, with all injected nude mice developing tumors by 6 days post implantation. The tumor-bearing animals were randomly selected and sorted into five groups (5 mice/

group). Group 1 was the reference control treated with vehicle (0.9% saline) only. Group 2 was the positive control treated with paclitaxel (Taxol) at 12.5 mg/kg. Groups 3−5 were treated with L-DOS47 at doses of 7, 35, and 175 μg/kg, respectively. Each animal from the test and reference control groups received three doses by injection scheduled at days 14, 18, and 22, while the positive control group received paclitaxel by injection on days 14, 18, 21, 25, and 28. Injection was administered intravenously via the tail vein. Tumor size was measured using a magnifying lens with a measurement grid etched into the glass. The length (L) and width (W) of any tumors that developed were measured in millimeters. The tumor volume in mm^3 was calculated using the formula: $T_{vol} = (\pi L W^2)/6$.

ASSOCIATED CONTENT

ⓢ Supporting Information
LC-MS analysis of the tryptic digests of urease and L-DOS47, MS/MS analysis of antibody conjugation sites, susceptibility of various tumor cell lines to NH_4Cl, and apparent binding affinity of L-DOS47 and AFAIKL2 antibody. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00237.

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Notes
The authors declare the following competing financial interest(s): B. Tian, W. Wong, P. Kumar, K. Gaspar, and H. Chao are employees of Helix BioPharma Corp.

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ABBREVIATIONS USED

BSA, bovine serum albumin; DTT, dithiothreitol; DMF, dimethylformamide; EDTA, ethylenediamine tetra acetic acid; ESI, electron spray ionization; IgG, immunoglobulin; IPTG, isopropyl β-D-1-thiogalactopyranoside; LC-MS, liquid chromatography−mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, salt; MWCO, molecular weight cutoff; NHS ester, N-hydroxysuccinimide ester; m/z, mass-to-charge ratio; PBS, phosphate buffered saline; PES, phenazine methyl sulfate; RP-HPLC, reverse phase high-performance liquid chromatorgraphy; SDS, sodium dodecyl sulfate; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; TBS, tris buffered saline; Tris-Cl or Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride buffer; UF/DF, ultrafiltration/diafiltration; UPLC, ultra performance liquid chromatography

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